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Production and application of a monoclonal antibody based peroxidase conjugate for detection of chicken IgG antibodies in ELISA

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Abstract

Viral infections are the cause of great economic losses in the poultry industry. Development of appropriate reagents and serological diagnostic kits will help to control these infections. The aim of this study is to prepare a peroxidase labeled anti-chicken IgG using a MAb (5B8) against chicken IgG, for detection of chicken antibodies in ELISA. Hybridoma cells producing the MAb 5B8 were cultured in RPMI 1640 medium and the MAb was purified from the cells supernatant using a sepharose matrix column, sensitized with chicken IgG. The purified MAb was labeled with Horseradish peroxidase (HRP) by periodate treatment. The peroxidase labeled MAb was compared with a commercial polyclonal product for detection of chicken antibodies against avian influenza virus nucleoprotein in ELISA. Therefore, type A recombinant nucleoprotein influenza virus was used as the antigen and chicken sera prepared from healthy and influenza virus infected chickens were used as primary antibodies. The results showed that there is a strong and direct correlation (r =0.972) between the optical densities of a commercial anti-chicken IgG and the prepared conjugate**.** In conclusion, the conjugated MAb is appropriate for the development of serological diagnostic tests for poultry infections.

Keywords: Chicken, IgG, Monoclonal antibody, Peroxidase conjugate

Introduction

Despite advances in diagnosis and treatment of infectious diseases, viral agents are still major threats to the poultry industry. Detection of antibodies against viral agents in chicken sera is very important for screening, monitoring and control of viral diseases. Presently, immuno enzymatic assays, in the form of commercial ELISA kits, are the most common serological tests for diagnosis of infectious diseases of poultry. In all of these kits, a secondary antibody labeled with an enzyme is used to detect the whole molecules of chicken IgG. Anti-chicken IgG secondary antibodies in the commercial kits are frequently the polyclonal antibodies produced in rabbits or goats. These polyclonal antibodies recognize several epitopes on the whole molecules of chicken IgG. However, mouse monoclonal antibodies (MAbs) against chicken IgG can also be used as secondary antibodies in ELISA (Myers *et al*., 1989). The major advantages of monoclonal antibodies compared to polyclonal antibodies are their homogeneity, consistency and higher specificity. Due to their advantages, enzyme conjugated MAbs could be more suitable than the polyclonal reagents for being used in the diagnostic tests (Leenaars and Hendriksen, 2005; Lipman *et al*., 2005; Pandey, 2010).

Today, chickens have become the preferred animal for production of antibody against mammalian antigens. This is due to their phylogenetic distance from their mammalian counterparts (Wooley and Landon, 1995; Schimizu *et al*., 1995; Hodek and Stiborova, 2003; Raj *et al*., 2004; Narat *et al*., 2004). Therefore, an enzyme conjugated MAb against chicken IgG could also be a valuable tool in this field of research.

The aim of the present study is to conjugate an anti-chicken IgG monoclonal antibody with peroxidase and to assess the application of the conjugate for the diagnosis of Influenza virus infection in chickens by ELISA.

Materials and methods

Culture of hybridoma cells

A hybridoma clone (5B8) (Fig. 1) producing monoclonal antibody against chicken IgG was previously produced by Seyfi Abad Shapouri (Seyfi Abad Shapouri, 2011). Hybridoma cells were cultured in 500 ml RPMI 1640 medium (Gibco, Scotland) supplemented with 10% of Fetal Bovine Serum (Gibco, Scotland). When the culture medium turned yellow, the supernatant was harvested and stored at -20°C until purification of the MAb.

Purification of the MAb 5B8 by Affinity chromatograghy

4B Sepharose beads (1 gram, sigma, United States) were re-suspended in 1 mM HCL and were transferred onto a filter paper placed in a sintered glass funnel. Sepharose beads were washed sequentially with 200 to 500 ml of 1 mM HCL and 200 to 500 ml of the coupling buffer (19.5 g/l $NaH_2PO_4.2H_2O$ and 17.75 g/l NaH_2PO_4, pH 8.3). After washing, the prepared gel (swollen beads) was transferred to a 50 ml plastic tube and was mixed with 1 ml of purified chicken IgG (17 mg/ml), and it was prepared according to a previously described method (Bhansuhali *et al*., 1994). The mixture was incubated overnight at 4°C on a rocker platform and then it was centrifuged at 250 ×g for 10 min. The supernatant was discarded and the gel was washed by the coupling buffer. In order to block the free sites on sepharose beads, 20 ml of 1 M ethanolamine, pH 8 were added and the beads were kept at 4°C for 5 hours. The beads were separated by centrifugation at 250 ×g for 10 min and the supernatant was discarded. Finally, the gel was washed by PBS and washing buffer (13.6 gr/l of $NaCooCH_3.3H_2O$, 29.2 g/l of Nacl, pH 4). A 2.5 cm column was packed with the prepared gel and was washed by 100 ml PBS. The hybridoma supernatant (as the source of the MAb 5B8) was centrifuged at 10000 X g for 30 min at 4°C and it was filtered through a 40 µm filter to

separate the cell debris. The cleared hybridoma supernatant was passed through the column at a flow rate of 1.5 ml/min. Finally, the column was washed with 50 ml PBS and the bound MAb was eluted with 15 ml of Gly–cl (3.75g/l Gly-cl and 8.77 g/l NaCl, pH 2.3). The eluate was collected in a tube containing 3.75 ml of neutralizing buffer (7.8g/l $Na_2HPO_4.2H_2O$ and 70.98 g/l Na_2HPO_4, PH 7.7) (Ausubel *et al*., 1992). The purified MAb was concentrated with saturated ammonium sulfate (761g/l) and its protein concentration was determined by spectrophotometer. Purification of the MAb was evaluated by SDS-PAGE analysis and titration by indirect ELISA as follows: A 96 wells ELISA plate (Karizmehr, Iran) was coated with the purified chicken IgG, and it was diluted in coating buffer (1.7 g/l Na_2Co_3, 2.86 g/l $NaHCO_3$) at the concentration of 1 µg/well. After an overnight incubation at 4°C, the plate was emptied and washed three times with PBST (0.05% Tween 20). The plate was blocked with PBST containing 5% skim milk for 3 hours at room temperature and was washed again as above. Thereafter, serial dilutions of the purified MAb 5B8 in PBST (1:200 to 1:1638400) were added in duplicate wells and were incubated for 1 hour at room temperature. The wells were washed as described above and 1:3000 dilution of a commercial HRP-labeled anti mouse IgG (Koma Biotech, Korea) was added to each well. Following incubation at room temperature for 1 hour, the plate was washed four times and the reaction was developed by adding chromogen substrate solution (20 μl 3, 3′, 5, 5′ of tetra methylbenzidine 1%, 980 μl 0.1M of acetate buffer pH 6, 1.6 μl H_2O_2 3% per ml). The reaction was stopped after 10 minutes by adding 50 μl of 0.1M HCl and the optical densities (ODs) were measured at 450 nm with an ELISA reader. In this ELISA, all reagents were applied at 50 μl/well except the washing and blocking buffers which were used at 300 μl/well (Ausubel *et al*., 1992).

Conjugation of the MAb 5B8 with HRP

Two hundred μl (2 mg/ml) of the purified MAb was dialyzed against 10mM sodium carbonate buffer (pH 9.5) at 4℃ for 24 hours. Five mg of HRP was dissolved in 500 μl of distilled water and was treated with 100μl of freshly prepared 0.15M sodium periodate for 30 min at room temperature. Treated enzyme was dialyzed overnight against 1mM sodium acetate buffer, pH 4.4, at 4℃. In order to adjust the pH of the peroxidase and antibody for optimum conjugation, 4 μl of 0.2M sodium carbonate buffer (pH 9.5) was added to 240 μl of the activated peroxidase before adding the antibody. Immediately 200μl of the purified MAb was added and the mixture was incubated for 3 hours at room temperature, on a rotary shaker. Then, 20μl of freshly prepared sodium borohydride (4 mg/ml) was added and was incubated for 2 hours at 4℃ with a brief shaking, every ten minutes. Peroxidase labeled antibody was dialyzed against PBS overnight with 2 changes of buffer. Finally, bovine serum albumin was added at a concentration of 0.2 mg/ml and the conjugate was stored at -20 ℃ (Wisdom, 2002). The efficiency of the labeling was tested by direct ELISA. Direct ELISA was principally performed as described above with the exception that the MAb 5B8, presumed to be labeled with HRP, was used as the conjugate. Another difference was in the serial dilutions of the MAb which were prepared from 1:500 to 1:32000.

Evaluation of the HRP-labeled MAb for detection of antibodies against avian influenza virus by indirect ELISA

ELISA plate was coated by recombinant nucleoprotein (NP) of type A influenza virus (1μg/well) (Jaidari, 2011), overnight at 4°C. The plate was washed three times with PBST and was blocked with 5% skim milk in PBST, for 2 hours at room temperature. After washing the plate as above, 28 chicken sera diluted 1:100 in PBST were added in duplicate wells. Sera included 14 samples collected from broiler chickens infected with H9N2 serotypes

of influenza virus and 14 samples obtained from broiler chickens non-vaccinated and non-infected with influenza virus. The plate was incubated for 1 hour at room temperature and then washed three times with PBST. Thereafter, 1:1000 dilution of the prepared conjugate in PBST and 1:5000 dilution of a commercial anti-chicken IgG conjugate (Sigma, USA) were added, each in two wells for each serum. After 45 minutes incubation at room temperature and four washes with PBST, the reaction was developed with the chromogen-substrate solution and stopped with 0.1M HCl as described above.

Results

Purification of Chicken IgG and the MAb 5B8

As described above, chicken IgG was purified from chicken serum by the method described by Bhanushali *et al*., (1994), including precipitation of all serum proteins except IgG with Caprylic acid, followed by ammonium sulfate precipitation of IgG. The MAb 5B8 was purified by affinity chromatograghy, using 4B **S**epharose beads, sensitized with the purified chicken IgG. SDS-PAGE analysis of purified antibodies is shown in Fig.2.

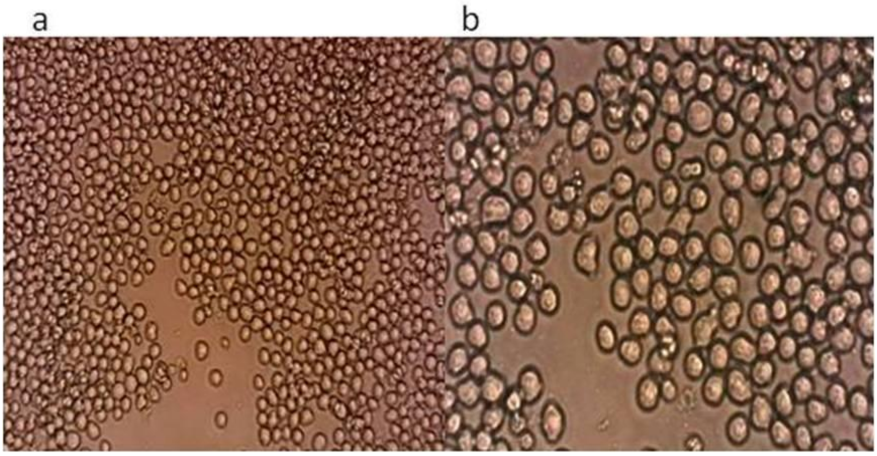


Figure 1. Hybridoma cells (a:100X, b:400X). 5B8 hybridoma cells, produced by fusion of SP2/0 myeloma cells and the spleen cells of a mouse immunized with chicken IgG.

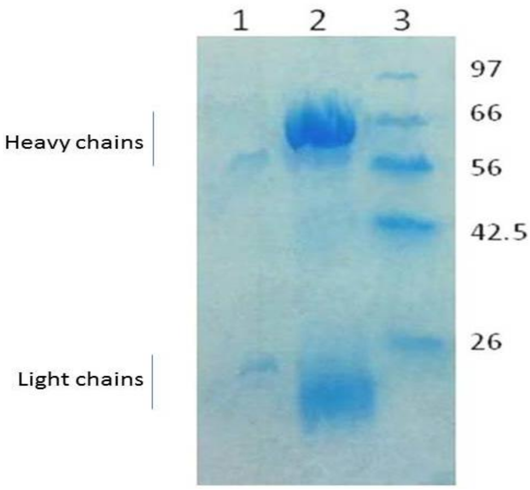


Figure 2. SDS-PAGE analysis of the purified MAb 5B8 (lane 1) and purified chicken IgG (lane 2). Lane 3 represents standard molecular weights in kDa. Positions of heavy and light immunoglobulin chains are indicated at the left.

Evaluation of the purified MAb 5B8 and the HRP conjugated MAb by titration in ELISA

The MAb 5B8, purified by affinity chromatography was evaluated by ELISA, using the purified chicken IgG (Fig. 2) as the antigen. The results showed that the purified MAb reacted with chicken IgG, up to a dilution of 1:204800. Optical density values obtained from the reaction of serial dilutions of the purified MAb with chicken IgG are indicated in Fig.3.

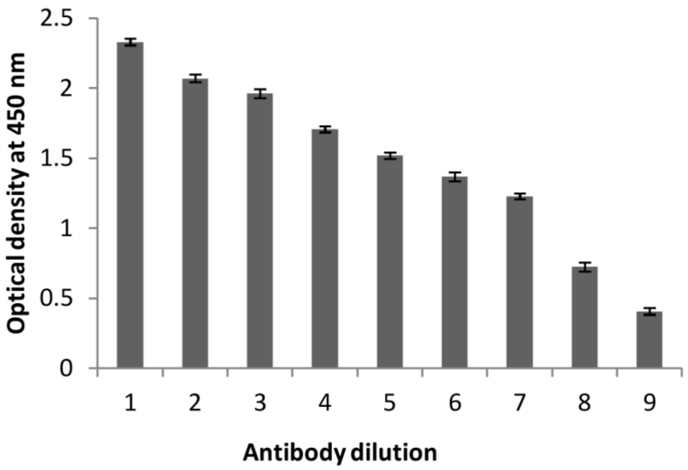


Figure 3. Optical density values of the reaction of serial dilutions of the purified MAb 5B8 with chicken IgG in ELISA. Twofold dilutions of the MAb (1:800 to 1:204800) are shown by numbers 1 to 9, respectively.

To determine the titer of the HRP-conjugated MAb by ELISA, serial dilutions (1:500 To 1:32000) of the conjugated MAb were added to wells of an ELISA microplate coated with chicken IgG and also to wells without chicken IgG as the control. As it is indicated in Fig.4, conjugated MAb reacted with chicken IgG up to a dilution of 1:32000, while any significant reaction was not observed in the control wells.

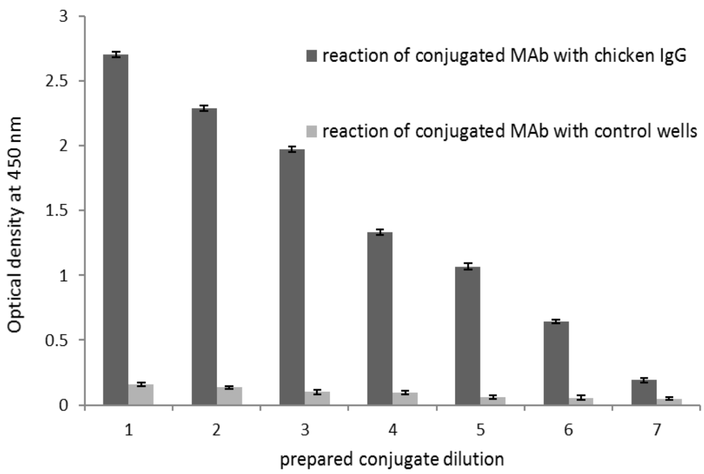


Figure 4. Optical density values of the reaction of serial dilutions of the conjugated MAb with chicken IgG and control wells (without chicken IgG) in ELISA. Each dilution was tested in duplicate and the mean OD value was presented. Twofold serial dilutions of the prepared conjugate (1:500 to 1:32000) are shown by numbers 1 to 7, respectively.

Comparison of the HRP-conjugated MAb with a commercial HRP-labeled anti chicken IgG for detection of antibodies against influenza virus NP in broiler chicken sera

Fourteen serum samples were collected from influenza virus infected broiler chickens and 14 sera from non-infected and non-vaccinated chickens were tested by both conjugates in ELISA. ELISA plate was coated with the recombinant NP of H9N2 serotype of avian influenza virus. Optical density values obtained by the conjugates are shown in Fig.5. A significant correlation was observed between the results of the commercial and the prepared conjugates. Distribution of tested serum samples based on the Optical density values obtained by both conjugates are shown in Fig.6. The results indicate a correlation coefficient of 0.972, $p<0.001$ between the OD values obtained by the commercial and the prepared conjugates.

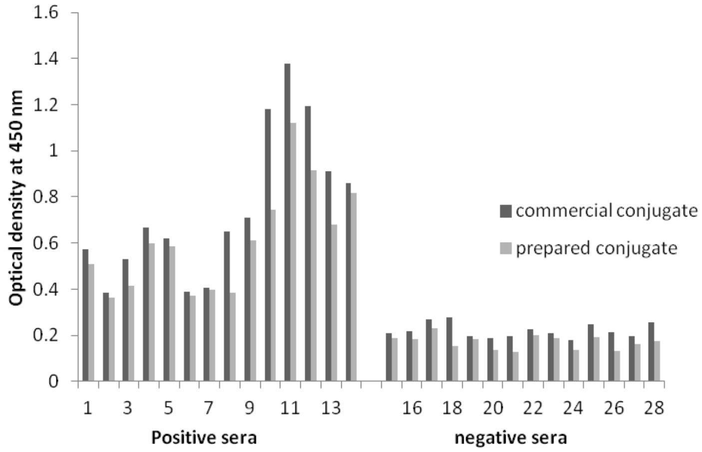


Figure 5. Optical density values of 14 positive sera, collected from influenza virus infected birds and 14 negative sera, obtained from non-infected and non-vaccinated birds, in NP-ELISA, using a commercial and the prepared conjugate. Each serum was tested in duplicate and the mean OD values were compared.

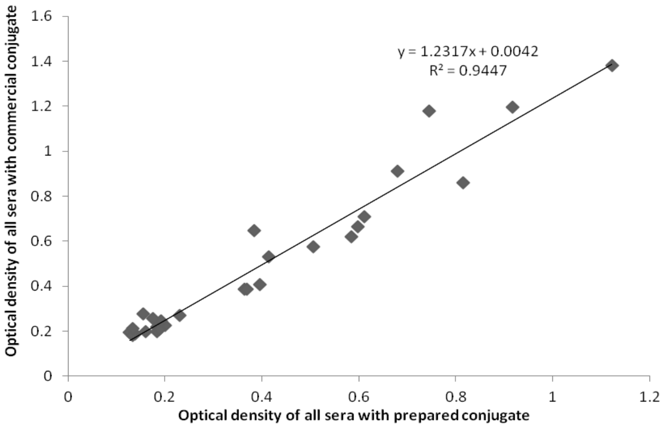


Figure 6. Correlation between the OD values of the tested serum samples of Figure 5, obtained by the commercial and prepared conjugates.

Discussion

Monoclonal antibodies have advantages of homogeneity, consistency and higher specificity compared to polyclonal antibodies. Nevertheless, so far a few studies have reported the application of enzyme conjugated MAbs against chicken IgG in indirect ELISA. Myers *et al*., (1989) developed an immunoglobulin class-specific ELISA to quantify antibodies against avian rotavirus.

Erhard *et al*., (1992) reported the development of specific ELISA for the quantitative and qualitative assay of chicken Ig isotypes G, M, and A using monoclonal antibodies. In 2004, Narat *et al*., produced several monoclonal antibodies against chicken IgG. Application of these antibodies in blotting immunological assays showed that one of the produced antibodies was very potent as a secondary antibody and was able to detect chicken antibodies against different pathogens. Raj *et al.,* (2004) produced several MAbs against chicken IgG and used one of them as a capture antibody in ELISA to quantify IgY purified from egg yolk.

Regarding the development of poultry industry in Iran and the necessity of access to reagents for diagnosis of infectious diseases of these animals, we recently produced an anti-chicken IgG monoclonal antibody (5B8) (Seyfi Abad Shapouri, 2011). The present work was performed to purify and label the MAb 5B8 with HRP and to assess this reagent, as anti-chicken IgG conjugate by ELISA. Antibody was purified by a sepharose column sensitized with chicken IgG. Indirect ELISA showed that the purified preparation had a high titer (1/204800) against chicken IgG.

Of the two enzyme labeling methods, namely treatment with sodium periodate (Nakane and Kawaoi, 1974) or with glutaraldehyde (Avarameas, 1971), we applied the periodate treatment for coupling of HRP to the purified monoclonal antibody in this study. Conjugates prepared with glutaraldehyde treatment have been reported to be more stable than those prepared by sodium periodate treatment. However, there is evidence that conjugates prepared by sodium periodate treatment might be more reactive (Tresca *et al*., 1995; Shepherd and Dean, 2000; Hamed *et al*., 2009). Therefore, as we expected the prepared conjugate was highly reactive (up to 1:32000 Dilution) with chicken IgG in ELISA. Although the ELISA titer of the conjugated MAb was sufficient for its application in the indirect ELISA its titer was significantly lower than the titer of the purified MAb before labeling (1:204800). Lower titer of the conjugated MAb could be due to efficiency of labeling protocol or the effect of linking of HRP molecules to MAb, similar to the effect of linking fluorochromes or radionuclides which may alter antibody binding (Lipman *et al*., 2005).

Comparison of the reactivity of this conjugate with a commercial product for detection of chicken antibodies against the nucleoprotein of avian influenza virus indicated that there was a significant correlation between the results obtained by the two conjugates. Based on the results of the indirect ELISA, the sensitivity of the commercial product which is a polyclonal anti-chicken IgG appears to be higher than that of our labeled MAb. This could be explained by the fact that the commercial polyclonal product recognizes more epitopes on the whole molecule of IgG, while the MAb based conjugate reacts with a single epitope on each IgG molecule. Although, this limitation can lead to a lower sensitivity it is the basis of the remarkable specificity of MAbs. As we found in this study, the lower sensitivity of the MAb could also be overcome in part by using a convenient dilution of the reagent.

In conclusion and based on our results, we hypothesize that the MAb based conjugate prepared in our study is appropriate for indirect ELISA. Therefore, the commercial polyclonal conjugates in diagnostic and research applications can be substituted with the conjugated MAb.

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References

Ausubel, FM; Brent, R; Kingston, RE; Moore, DD; Seidman, JGand Smith, JA (1992) Short protocols in molecular biology. John Wiley and Sons, New York.

Avrameas, S and Ternynck, T (1971). Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochemistry* **8**, 1175-1179.

Bhanushali, JK; Gilbert, JM and Mcdougald, LK (1994). Simple method to purify chicken immunoglobulin G. *Poultry Science* **73**, 1158- 1161.

Erhard, MH; Von Quistrop, I; Junglling, A; Kaspers, B; Schmidt, P and Kuhlmann, R (1992). Development of specific enzyme-linked immunosorbent antibody assay systems for the detection of chicken immunoglobulins G, M, and A using monoclonal antibodies. *Poultry Science* **71**, 302-310.

Hamed, RR; Mohamed, T; EL Hakim, MA and Gad, AA (2009). Biochemical studies on the conjugation of antibodies with turnip peroxidase using two different methods. *Journal of Biological Research-Thessaloniki* **12**, 173-186.

Hodek, P; and Stiborova, M (2003). Chicken Antibodies Superior Alternative for Conventional Immunoglobulins. *Proceedings of the National Academy of Sciences, India* **69**, 461-468.

Jaidari, A (2011). Cloning and expression of the NP gene of H9N2 virus in *Escherchia coli*, Shahid Chamran University, Ahvaz, Iran.

Leenaars, M and Hendriksen, CF (2005). Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. *Institute for Laboratory Animal Research* **46**, 269-279.

Lipman, NS; Jackson, LR; Trudel, LJ and Weis-Garcia, F (2005). Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *Institute for Laboratory Animal Research* **46**, 258 -268.

Myers, TJ; Schat, KA and Mockett, AP (1989). Development of immunoglobulin class-specific enzyme-linked immunosorbent assays for measuring antibodies against avian rotavirus. *Avian Diseases* **33**, 53-59.

Nakane, PK and Kawaoi, A (1974). Peroxidase-labeled antibody, a new method of conjugation. *Journal of Histochemistry and Cytochemistry* **22**, 1084-1091.

Narat, M; Bicek, A; Vadnjal, R and Bencina, D (2004). Production, characterization and use of monoclonal antibodies recognizing IgY epitopes shared by chicken, turkey, pheasant, peafowl and sparrow. *Food Technology Biotechnology* **42**, 175-182.

Pandey, S (2010). Hybridoma technology for production of monoclonal antibodies. *International Journal of Pharmaceutical Sciences Review and Research* **1**, 88-94.

Raj, GD; Latha, B; Chandrasekhar, MS and Thiagarajan, V (2004). Production, characterization and application of monoclonal antibodies against chicken IgY. *Veterinary Archives* **74**, 189-199.

Schimizu, M; Watanabe, A and Tanaka, A (1995). Detection of high molecular weight mucin like glycoprotein–A (HMGP-A) of human milk by egg yolk antibody. *Bioscience Biotechnology and Biochemistry* **59**, 138-142.

Seyfi Abad Shapouri, MR (2011). Production of monoclonal antibody against chicken IgG, Research Project number 13. Shahid Chamran University, Ahvaz, Iran.

Shepherd, P and Dean, C (2000); Monoconal antibody, Oxford university press, New York.

Tresca, JP; Ricoux, R; Pontet, M and Engler, R (1995). Comparative activity of peroxidase-antibody conjugates with periodate and glutaraldehyde coupling according to an enzyme immunoassay. *Annales de biologie clinique* **53**, 227-231.

Wisdom, GB (2002). Horseradish peroxidase labeling of IgG antibody in The protein protocols handbook, Humana Press,

Totowa, New Jersey.
Wooley, JA and Landon, J (1995). Comparison of antibody production to human interleukin-6 (IL-6) by sheep and chickens. *Journal of Immunological Methods* **178**, 253-260.

تولید و کاربرد کونژوگه پراکسیداز آنتی‌بادی مونوکلونال به منظور تشخیص IgG مرغی توسط ELISA

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چکیده

عفونت های ویروسی عامل خسارت‌های اقتصادی بسیار زیادی در صنعت طیور هستند. تولید مواد و کیت های تشخیص سرولوژیک مناسب به کنترل این عفونت ها کمک خواهد نمود. هدف از مطالعه‌ی حاضر تهیه یک آنتی بادی نشاندار شده با پراکسیداز ضد IgG مرغ، با استفاده از یک آنتی بادی منوکلونال ضد IgG مرغ (5B8)، برای ردیابی آنتی بادی های مرغ در الیزا بود. برای این منظور تیره سلول هیبریدومای تولید کننده‌ی آنتی‌بادی مونوکلونال 5B8 در محیط RPMI 1640 کشت داده شد و آنتی بادی منوکلونال با استفاده از یک ستون حاوی سفارز حساس شده با IgG مرغ از مایع رویی سلول‌ها خالص شد. آنتی‌بادی خالص شده با روش پریودات با آنزیم پراکسیداز نشاندار گردید. آنتی‌بادی منوکلونال نشاندار شده با پراکسیداز از نظر شناسایی آنتی بادی ضد نوکلئوپروتئین ویروس آنفلونزا در الیزا با یک آنتی بادی پلی کلونال تجاری مقایسه گردید. بنابر این نوکلئوپروتئین ویروس آنفلونزا تیپ A به عنوان آنتی ژن و سرم های تهیه شده از مرغ های سالم و مرغ های آلوده به ویروس آنفلوآنزا به عنوان آنتی بادی اولیه مورد استفاده قرار گرفتند. نتایج بدست آمده نشان داد که یک همبستگی قوی و مستقیم (r =0.972) بین دانسیته های نوری آنتی بادی تجاری ضد IgG مرغ و آنتی بادی نشاندار شده وجود دارد. در نتیجه آنتی بادی منوکلونال نشاندار شده برای طراحی کیت‌های تشخیص سرمی طیور مناسب می باشد.

واژگان کلیدی: مرغ، IgG، آنتی‌بادی مونوکلونال، کونژوگه پراکسیداز